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AWARD NUMBER: W81XWH-08-1-0583

TITLE: Multimodal Imaging of Pathophysiological Changes and Their Role in Development of Breast Cancer Brain Metastasis

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REPORT DATE: September 2009

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
1 September 2009	Annual	1 Sep 2008 – 31 Aug 2009

4. TITLE AND SUBTITLE

Multimodal Imaging of Pathophysiological Changes and Their Role in Development of Breast Cancer Brain Metastasis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-08-1-0583

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Texas Southwestern Medical Center at Dallas
Dallas, TX 75390

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Brain metastasis represents a poor prognosis and is frequently the cause of death in breast cancer patients. Tumor microcirculation and oxygenation play important roles in malignant progression and metastasis, as well as response to various therapies. Understanding of hypoxia development and its relationship with blood brain barrier (BBB) during intracranial tumor growth will be crucial for clinical management of breast cancer brain metastasis. We have developed a MRI approach based on an interleaved T2*- and T1-weighted MRI sequence, which will provide information of both tumor vascular and tissue oxygenation. Moreover, by introducing hypoxia reporter gene (HRE-luciferase) into breast tumor lines, we will be able to use bioluminescence imaging to monitor hypoxia initiation and development of intracranial tumors. We will also correlate BBB function based on dynamic contrast enhanced (DCE) MRI with tumor hypoxia. We believe that integration of MRI and BLI will provide temporal and spatial information of tumor hypoxia evolution. Tumor hypoxia leads to resistance to anticancer therapies, in particular radiation, which is perhaps the most important treatment modality in our current armamentarium for brain metastasis. A combination of radiation with hypoxia modifier, 2-methoxyestradiol, on brain metastases will be evaluated by in vivo imaging.

15. SUBJECT TERMS
Breast cancer brain metastasis, hypoxia, Magnetic resonance imaging, Optical imaging, irradiation

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	16	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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Introduction:

Brain metastasis represents an important cause of morbidity and mortality. Clinically overt brain metastases occur in approximately 10 ~ 15% of patients with breast cancer (1, 2). The incidence of brain metastasis seems to have increased over the past decade, and may be the paradoxical result of effectiveness of drugs on primary breast cancer. Perhaps even more alarming are the growing numbers of breast cancer patients who die from complications related to brain metastasis, at a time when systemic disease is under good control. In part, this may be due to the fact that chemotherapeutic agents that show efficacy against systemic disease, may have poor penetration of the blood-brain barrier (BBB), which means that breast cancer metastasis in the brain may remain untreated and inaccessible to conventional chemotherapeutics (3-5).

Tumor microcirculation and oxygenation play important roles in malignant progression and metastasis, as well as response to various therapies. In particular, radiotherapy, and possibly some anticancer drugs, are less effective in hypoxic tumors (6, 7). There is little knowledge about tumor hypoxia during intracranial development of brain metastasis. We hypothesize that tumor hypoxia is major driving force for progression of breast cancer brain metastasis and represents a critical target for therapeutic strategies. Traditionally, pathophysiological and biological studies of brain tumor models involve sacrificing animals at different time points, and thus require a large number of animals. *In vivo* imaging promises greater efficiency since each animal serves as its own control and multiple time points can be examined sequentially. In addition to anatomic information, magnetic resonance imaging (MRI) has been increasingly applied to studying tumor pathophysiology. Blood Oxygenation Level Dependent (BOLD) MRI based on T_2^* contrast, deoxyhemoglobin, is sensitive to tumor vascular oxygenation. Recently, several studies have suggested a possibility of assessing tissue oxygenation by direct T_1 shortening due to oxygen molecule (8, 9). We have developed a MRI approach based on an interleaved T_2^*- and T_1-weighted sequence, which provides information of both tumor vascular and tissue oxygenation. Here, we plan to apply this new MRI approach to evaluating tumor hypoxia among various breast tumor lines growing intracranially.

Bioluminescence imaging (BLI), based on *in vivo* expression of luciferase, the light emitting enzyme of the firefly, is being rapidly adopted in cancer research. Luciferin, the substrate of luciferase, crosses the cell membrane and penetrates the intact BBB after injection in mice (10, 11). Several studies have demonstrated that the BLI is capable of tracking intracerebral neural cell migration (12) or monitoring intracranial tumor growth and its response to treatment (10), (13). Here, we propose to introduce a hypoxia reporter system, Hypoxia responsive element-luciferase (5HRE-luc), to various breast cancer cells. Hypoxia Inducible Factor-1alpha (HIF-1α) activity will be monitored via *in vivo* BLI by using a luciferase reporter gene under the regulation of an artificial HIF-1-dependent promoter, 5HRE (14, 15). Integration of MRI and BLI will provide temporal and spatial information of tumor hypoxia evolution.

Body:

The Statement of Work in this project had two major tasks:

Task 1. Establish mouse xenograft models of breast cancer brain metastasis and evaluate differential biological features among various breast cancer cell lines (Months 1-8):

a. Stable transfection with a retrovirus vector expressing firefly luciferase and a permanent, high expressing transfectant will be selected.

b. Establishment of intracranial implantation metastasis model and comparison and selection of cell lines that are able to grow in brain from ~ 5 human breast cancer cell lines, available at UTSW Hamon Cancer Center.

c. Bioluminescent imaging for *in vivo* detection of brain metastases and follow up of tumor growth.
d. MRI monitoring of brain metastasis growth *in vivo* and evaluation of dynamic change in tumor perfusion and permeability (blood-brain barrier) during tumor growth.
e. Correlation of imaging findings with histological and ultrastructural studies of markers of perfusion and permeability, tumor hypoxia, angiogenesis and feeding vessels.

MDA-MB231 cell line has been stably transfected with the firefly luciferase gene. Utilizing the MDA-MB231-luc cells, intracranial tumor model has been established in athymic nude mice (Fig. 1). Bioluminescence imaging successfully detected intracranial signal, which increased in light intensity over time (Figs. 1 and 2).

Day 0 **Day 4** **Day11**

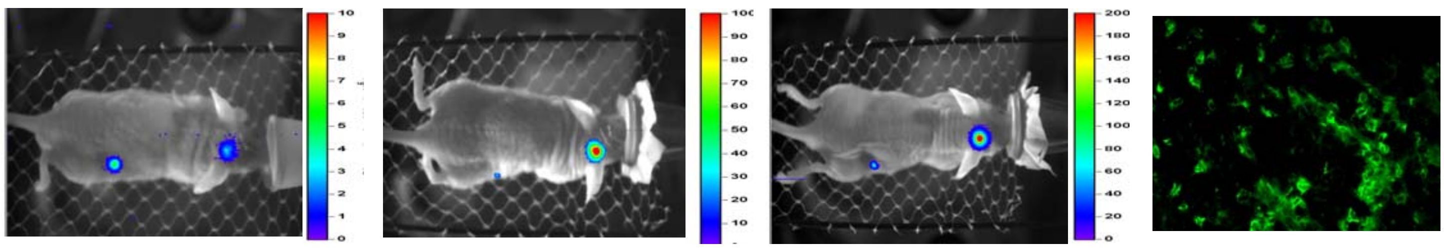

Fig. 1 BLI follow-up intracranial growth of human breast cancer MDA-MB-231-luc.
5 x 10^4 cells in 3 μl of the mixture of PBS and Matrigel were injected into the area of right caudal nucleus through a ~ 1 mm burr hole. As a control, equal numbers of cells were injected subcutaneously in right flank. The planar BLI study was performed immediately after injection (day 0) and followed on day 4 and 11. A weak, but clear intracranial signal was observed immediately after injection, which increased significantly with time. In contrast, the s.c. tumor showed typically latent pattern of growth. NOTE: different scale was used for each image (Day 0: 0-10; Day 4: 0-100; Day 11: 0-200 x 10^6 photons/sec/cm^2). Immunostaining against luciferase showed extensive expression of luciferase in tumor tissues dissected from brain.

Anatomic MRI has also been applied to monitor tumor growth (Fig. 3). Correlation with histological study has been performed (Figs. 2 and 3). In addition to anatomic MRI, functional MRI has been applied to monitoring tumor perfusion and permeability (Fig. 4) and maps of BBB permeability have been generated based on DCE MRI (Fig. 5)

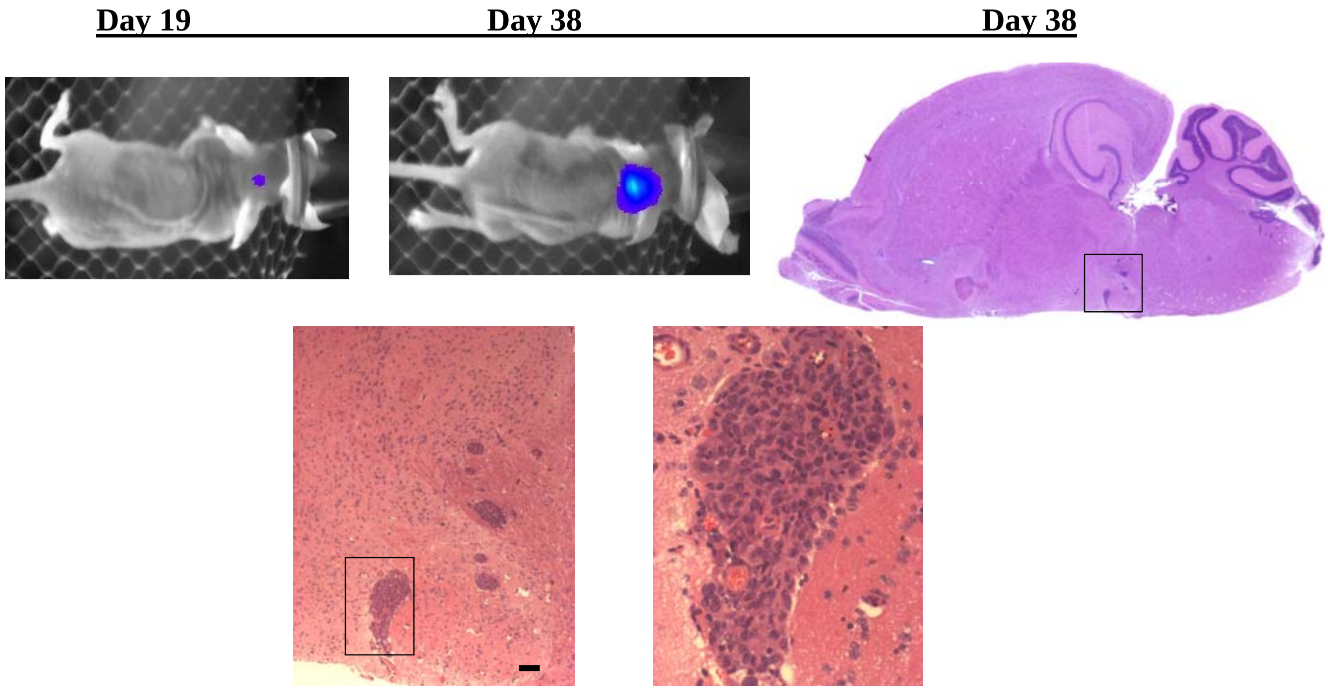

Fig. 2 Intracranial implantation of a small number of breast MDA-MB-231-luc cancer cells. BLI detected intracranial signal at Day 19 after 5,000 cells implantation and monitored tumor growth till Day 38. Histological H&E staining suggests several micrometastases located at area of corpus striatum. An enlarged image (Bottom right) of the tumor containing area in the box of Top right image clearly showing separately located tumor nodules. Further enlargement of the tumor nodules, shown in the box of bottom left, reveals densely populated tumor cells and development of intratumoral vasculature.

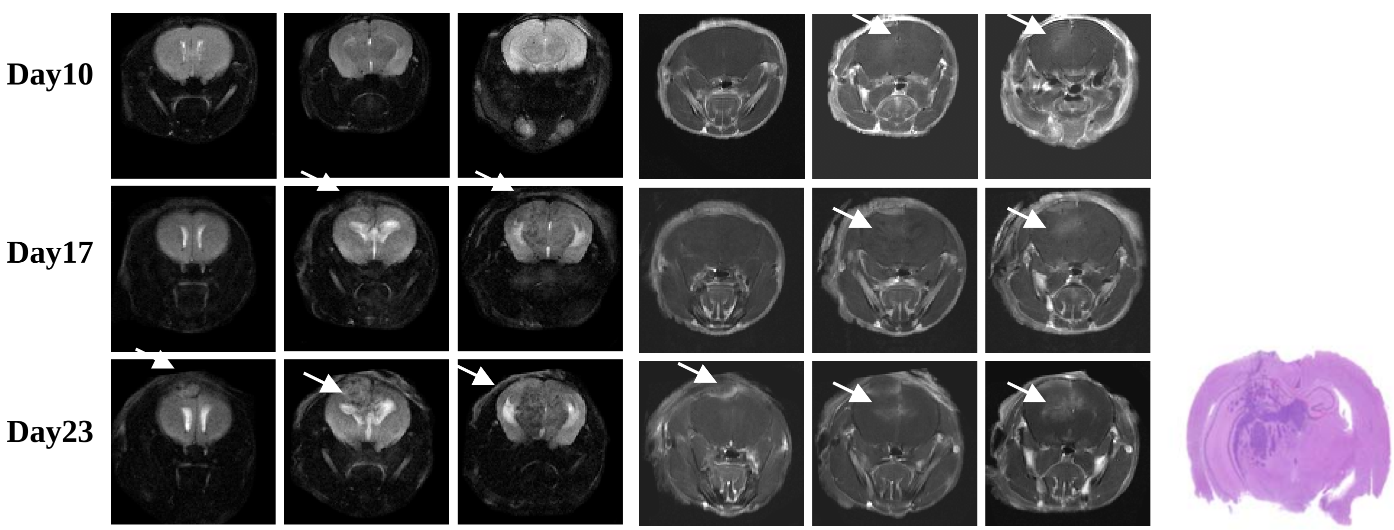

Fig. 3 MRI follow-up of the intracranial tumor growth. MRI started on Day 10 for the mouse shown in Fig. 1, with initial 5 x 10^4 MDA-MB231-luc cell implantation. Three contiguous coronal slices (thickness = 1.5 mm) selected from a series of MRI identified the tumor lesion in the caudal area of right hemisphere (arrows). An iso- or low-intensity lesion started to appear on T2-weighted images (arrows, left) on Day 17, accompanied by enlarged lateral ventricles and midline shift. Corresponding sections on T1-weighted images (right) 5 mins after i.v. bolus injection of the contrast Gd-DTPA highlight the tumor (arrows), indicating the BBB leakage. H&E staining confirms the tumor.

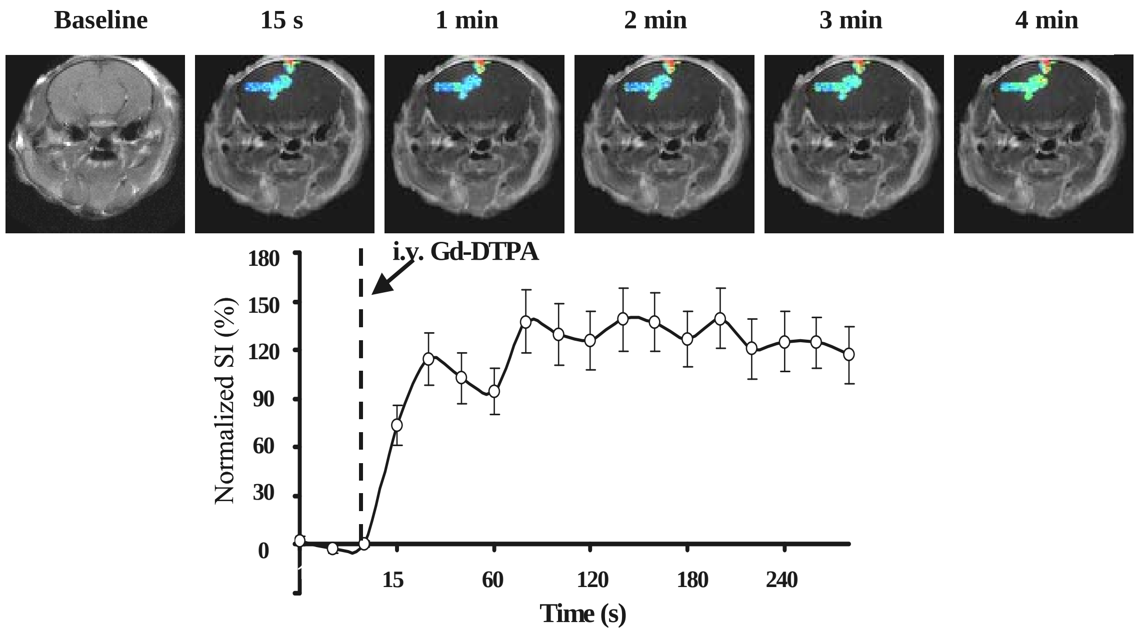

Fig. 4 DCE MRI evaluation of tumor perfusion/permeability. Top row: Dynamic maps of signal enhancement are overlapped on the T1-weighted image in the tumor 10 days after intracranial implantation. Heterogeneous signal enhancement was observed intratumorally. The subdural area with highest signal intensity, while accompanying tumor growing to further right side, the BBB disruption expanded. Bottom row: Signal intensity-time curve showed rapid increase in signal intensity immediately after Gd-DTPA infusion, which reached a plateau 1 min later.

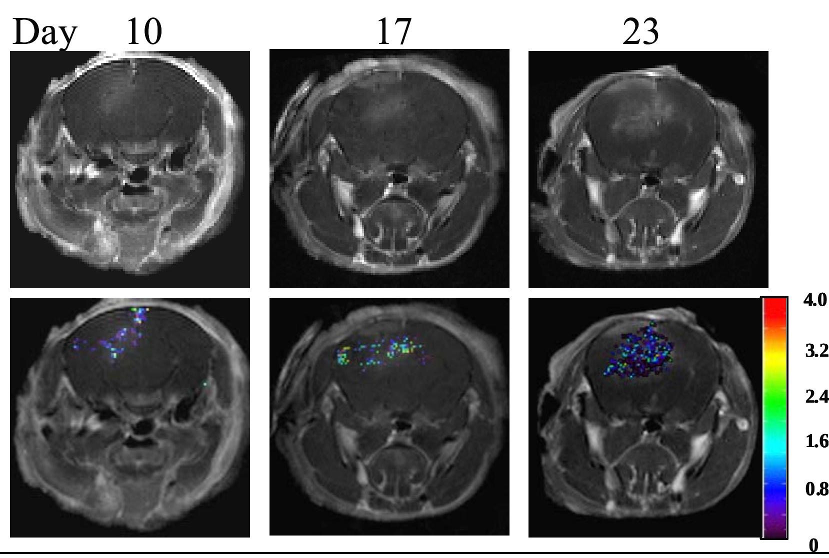

Fig. 5 Dynamic MRI evaluation of blood brain barrier (BBB) function in a mouse model of breast tumor brain metastasis. Sequential MRI scans were performed at different time points after intracranial tumor implantation. T_1-weighted contrast enhanced MRI showed a tumor growing intracranially (Top row). The BBB permeability is evaluated by calculating the constant of contrast exchange rate *Kep* based on dynamic contrast enhanced (DCE) MRI. The *Kep* maps overlapping on the anatomic images revealed development of BBB disruption and heterogeneity of BBB permeability within the tumor (Bottom row). This information will be useful for adjuvant chemotherapy or targeted molecular therapy.

While gene transfection and selection of stable luciferase clone for the 5 HCC lines are undertaken, we have preceded into works of Task 2.

Task 2. Multimodal imaging evaluation of intr acranial tumor hypoxia development and its correlation with blood brain barrier as well as aggressiveness of breast cancer brain metastasis (Months 9-24).

Progress in Task 2:

a. **The construct of HRE-ODD-luc h as been successfull y transfected into genome of MDA-MB231 cells. Stable transfect has been selected. In vitro both luminesence assays and biolumin escence i maging st udy have confirmed significantly highe r expression of luciferase in the cells incubated under h ypoxic condition (1 % O_2) compared to normoxia (Table 1. and Fig. 6).**

Table 1. Luminescence assays of MDA-MB231-HRE-ODD-luc cells

Oxygen concentration	Cell lysis no.	Relative light unit
normoxia (21%)	1	760658
	2	245590
	3	136654
	4	155754
	mean	324664
hypoxia (1%)	1	10220576
	2	30396726
	3	2850247
	4	2200730
	mean	11417069.8
Ratio (hypoxia/normoxia)		35.2

Note: equal number of cells (~ 300K) were lysed for each lysis. Significantly high (RLU) was detected in hypoxic cells ($p < 0.05$).

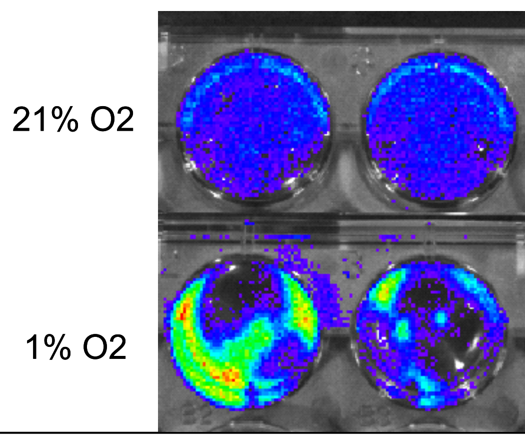

Fig. 6 *In vitro* confirmation of h ypoxia promoted light emission in MDA-MB231 cell line w ith stable HIF-1α-luc transfection. 300K cells cultured in 12-well dish were incubated under hypoxic (1% O_2 chamber) or normoxic (21% O_2) condition for 24 hr before BLI. Significant increase in signal intensity was observed in cells under hypoxic condition.

b. In vivo studies have been initiated. 5 x 10^4 MDA-MB231-HRE-ODD-luc cells were directly injected into caudal nuclear area of right side mouse brain. BLI was applied to monitoring temporal development of intratumoral hypoxia (Figs. 7 and 8).

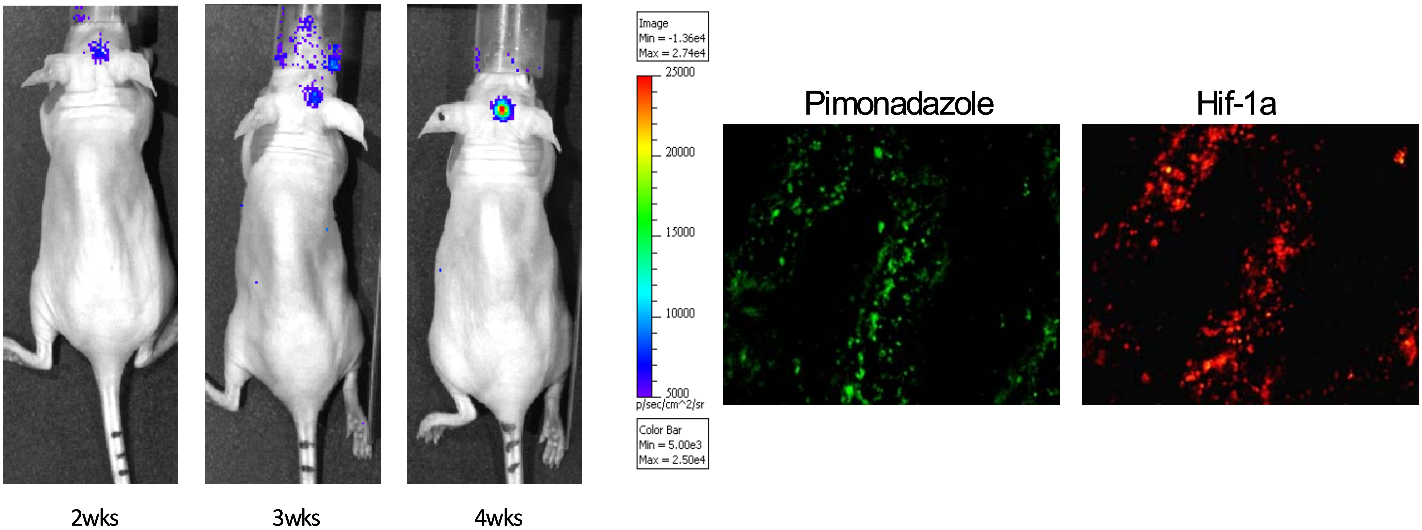

Fig. 7 *In vivo* detection of evolution of tumor hypoxia in MDA-MB231 cells with stable HIF-1α-luc transfection. 50K cells were injected into the right side brain of a nude mouse. A week signal was found 2 wks after, which increased in intensity in follow-up studies. Immunofluorescent staining of tumor tissue sections showed colocalization of pimonidazole and HIF-1α.

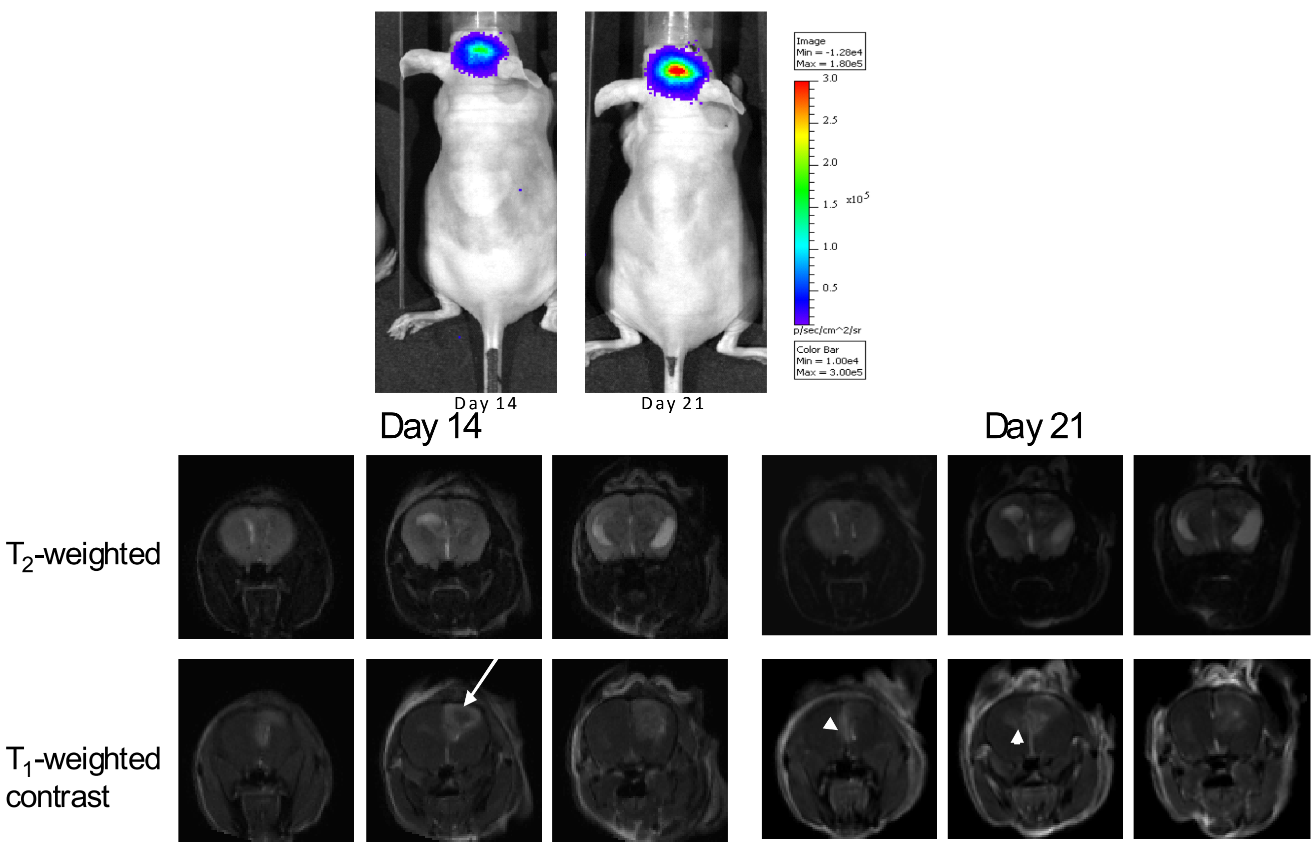

Fig. 8 *In vivo* BLI and MRI study of intracranial tumors. Tumor hypoxia based on the HIF reporter gene in a representative intracranial tumor of MDAMB231-HRE-ODD-luc was monitored by BLI. Corresponding MRI images of three consecutive slices on T2-weighted sequence on day 14 showed an iso or lower intensity region, which became hyperintensity on T1-weighted contrast enhanced images. The middle slice showed a ring shaped enhancement (arrow). On day 21, the tumor grew bigger and crossed the middle line to invade the left side brain (arrow heads). The distortion of lateral ventricle and accumulation of CSF became significant.

The major goal of this project is to integrate multiple parameters of tumor hypoxia and vasculature acquired by multimodal imaging to correlate with tumor aggressiveness and understand pathophysiological mechanism underlying the clinical benefits from antiangiogenic treatment. Thus, in addition to anatomic MRI, functional MRI of studying tumor vascular and tissue oxygenation and its correlation with tumor perfusion has been initiated. Interleaved T1-weighted (TOLD) and T2*-weighted (BOLD) sequence was used to assess tumor hypoxia. Dynamic susceptibility contrast (DSC) sequence was applied to study tumor perfusion (relative tumor blood volume, rTBV). More importantly, spatial correlation between TOLD, BOLD and rTBV was performed (Figs 9 and 10).

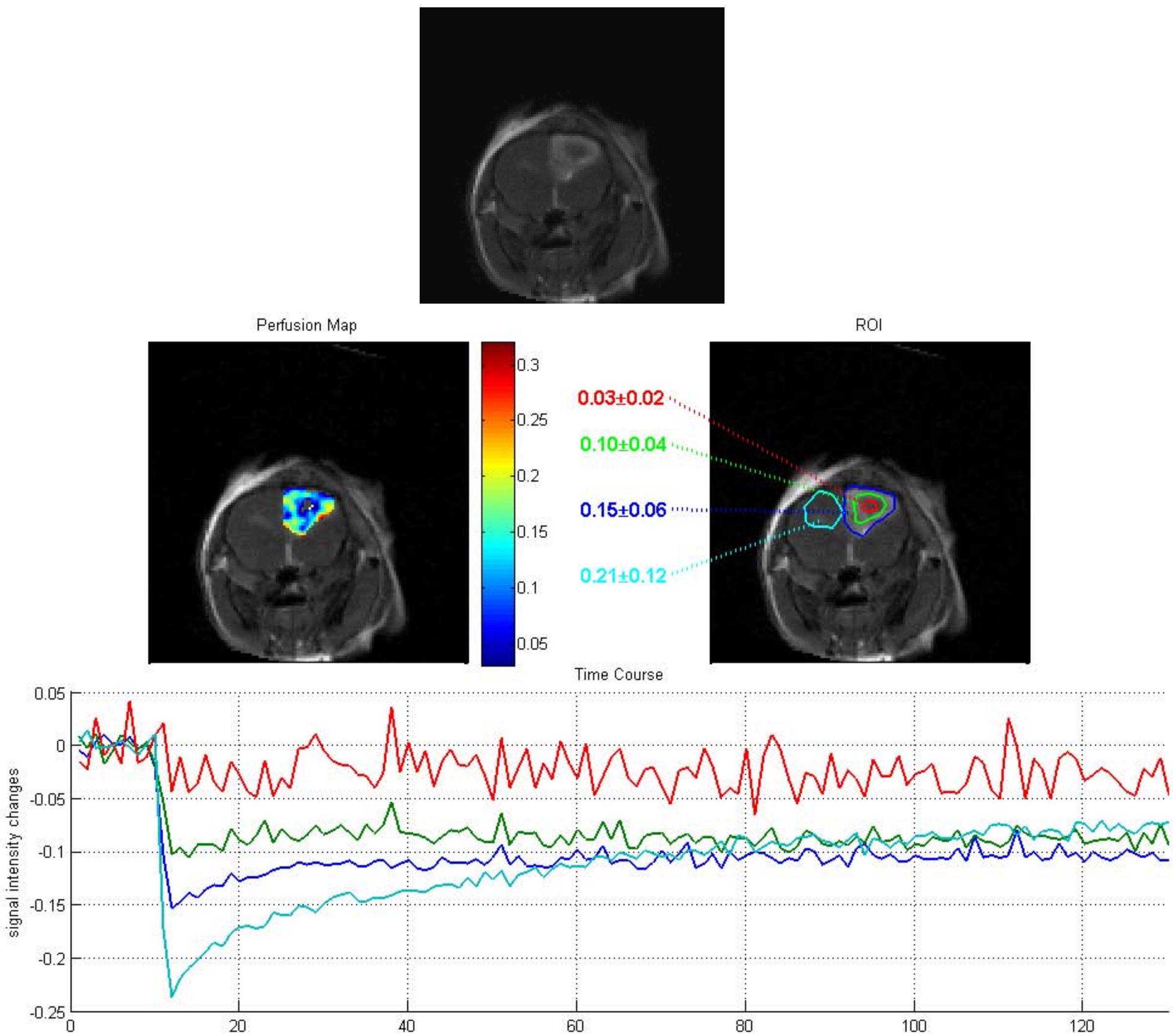

Fig. 9 MRI interrogation of tumor perfusion. DSC MRI was applied to study perfusion of the intracranial tumor, as shown in Fig. 8. T1-weighted contrast enhanced image showed highest ring-shaped enhancement in tumor periphery, while little or no enhancement was seen in tumor center. Based on this observation, 3 region of interest (ROIs) were selected to represent tumor periphery (between dark blue and green), tumor center (red) and the intermediate region (between green and red). rTBV (relative tumor blood volume) map was generated based on the time course of signal intensity change after a bolus injection of contrast agent, Gd-DTPA. In a good agreement with T1-contrast enhanced image, the highest rTBV was detected in tumor periphery. Time course curve of signal intensity change revealed the significantly deeper dip (first pass) in tumor periphery (blue) compared to the intermediate tumor (green). There was essentially no change in central tumor (red). Surprisingly, the contralateral normal brain region (light blue) showed deepest dip, indicating higher rCBV in normal brain than that in the tumor comprising of sluggish and nonfunctional vessels.

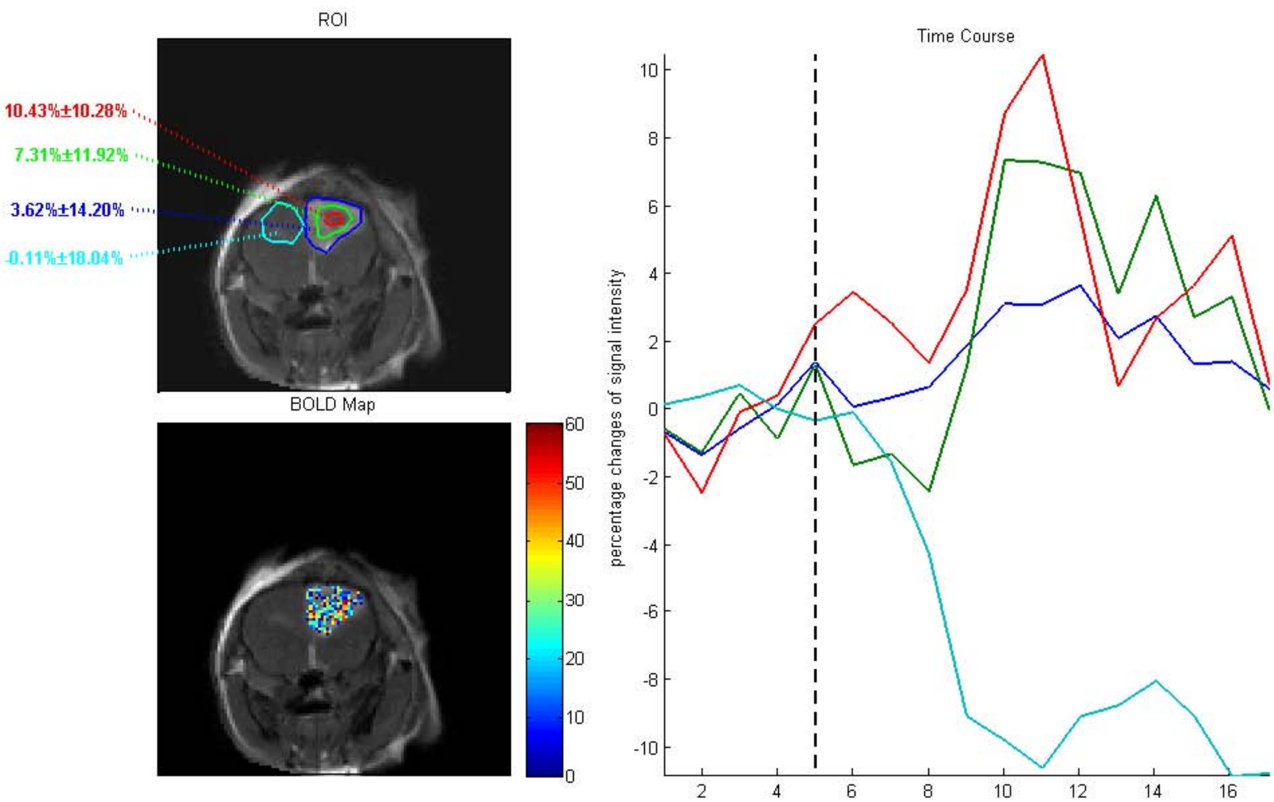

Fig. 10 BOLD MRI study of tumor blood oxygenation. The same tumor as well as the three ROIS as shown in the perfusion map in Fig. 9 was analyzed for BOLD effect induced by pure oxygen inhalation. In contrast to the perfusion pattern, which showed the highest rTBV in tumor periphery (Fig. 9), the tumor periphery had a much lower BOLD effect (< 4%, dark blue), compared to 7% increase in intermediate tumor (green) or even 10% increase in tumor center (red). The paradoxical observations may well suggest the intratumoral hypoxia gradients with severe hypoxia in tumor center, then intermediate tumor and lastly tumor periphery. The hypoxic tumor center responded most to oxygen, while relative better oxygenated tumor periphery had less response. The contrlateral normal brain, however, showed negative BOLD response, indicating decreased blood flow caused by vasoconstrictive effect of oxygen. BOLD map (left bottom) revealed intratumoral heterogeneous response.

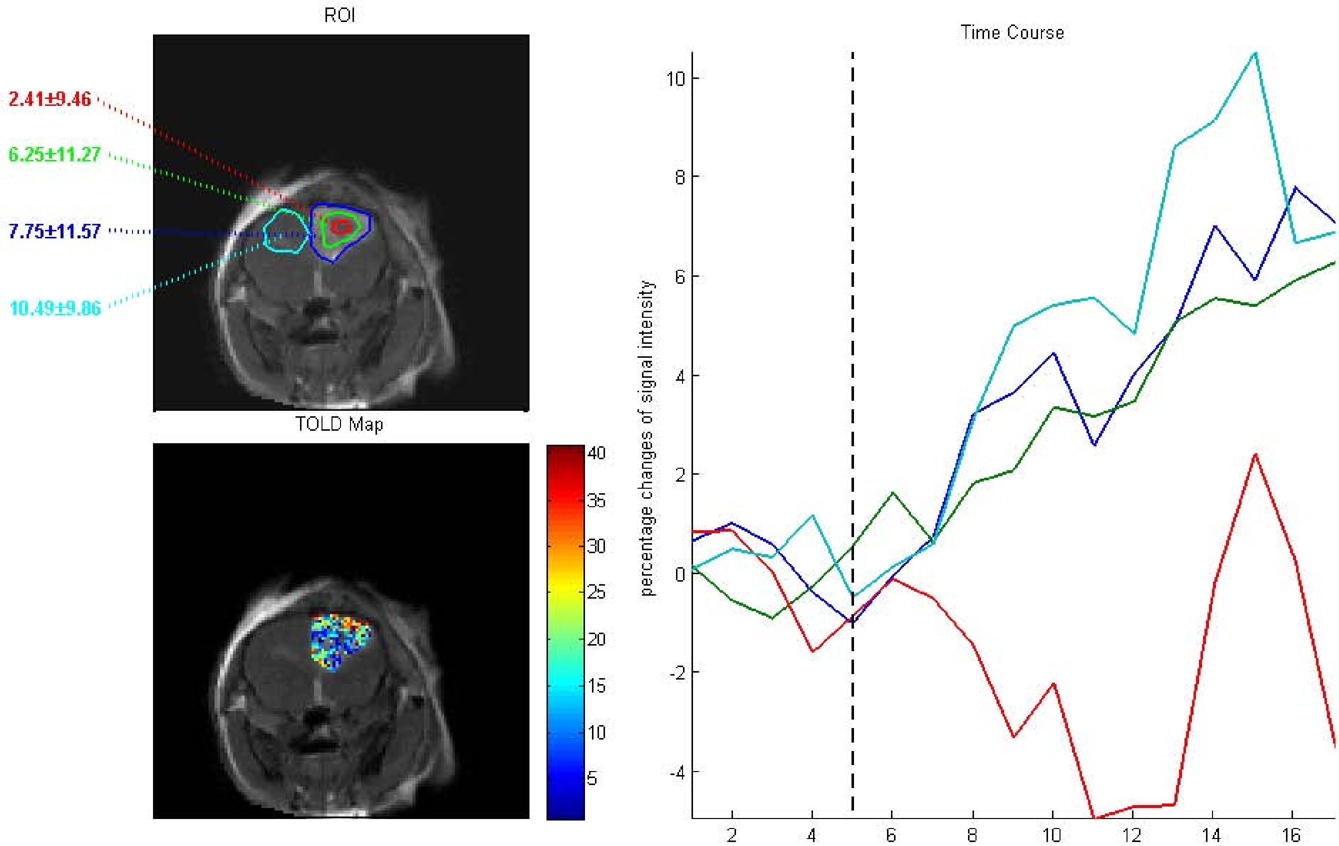

Fig. 11 TOLD MRI study of tumor tissue oxygenation. The same tumor regions as shown in Figs. 9 and 10 was analyzed for TOLD effect induced by pure oxygen inhalation. Gradual increase in T1-weighted signal intensity was found in tumor periphery (dark blue) and intermediate tumor (green) as well as the contralateral normal brain, while the tumor center showed first decrease and then modest increase in signal intensity. This explains well that TOLD assesses tissue oxygen (supply and consumption) while BOLD measures blood oxygen (supply only). Thus, these results may be interpreted that oxygen breathing results in increased oxygen tension in normal brain and tumor regions except tumor center, in which severely hypoxic tumor cells presumably have the highest oxygen consumption despite increased oxygen supply detected by BOLD effect. TOLD map (left bottom) showed heterogeneous regional response.

Key Research Accomplishments

- **Establishment of breast cancer brain metastasis in a mouse model.**

- **Successful application of in vivo BLI and MRI to monitoring intracranial development of breast cancer brain metastasis.**

- **Introduction of hy poxia reporter gene in br east cance r line and validation by in vitro and histological studies.**

MDA-MB231 cells were stably transfected with the HRE-ODD-luc construct.

- **In vivo assessment of tumor hypoxia by BLI monitoring of the hypoxia reporter gene, HIF-1 promoted luciferase expression.**

- **In vivo MRI study of tumor perfusion (rTBV, DSC MRI) and tumor oxygenation (BOLD and TOLD MRI).**

- **Spatial correlation between these MRI parameters is performed.**

Reportable Outcomes

Abstract (Published Conference Proceedings):

Heling Zhou, Li Liu, Kate Luby-Phelps, Debabrata Saha, Ralph Mason, **Dawen Zhao** Dynamic near-infrared optical imaging of 2-deoxyglucose uptake by intracranial tumors of athymic mice model. World Molecular Imaging Cogress. Montreal, Canada, Sep 2009.

Employment or research opportunity:

A PhD student, Heling Zhou, was recruited for this project. Ms. Zhou has great talents in imaging process and software development. An automated imaging processing software has been developed and applied to analyze coregistrational data of multiple MRI parameters.

Conclusion:

During the first year of this project, we have established a breast cancer line with stable transfection of hypoxia reporter gene. The breast cancer brain metastasis model has been established and in vivo imaging approaches have been applied to interrogate intracranial tumor hypoxia and vascular perfusion. A software system has been developed and automated to process imaging data and perform spatial correlation. Interesting preliminary data of in vivo imaging has been achieved and needs to be validated. Taken together, the first year research has built a strong foundation and will facilitate the research proposed in the project.

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Appendices

Presentation Number **0514**
Poster Session 3f: In Vivo Studies
September 26, 2009 / 16:00-17:30 / Room: 519

Dynamic near-infrared optical imaging of 2-deoxyglucose uptake by intracranial tumors of athymic mice

Heling Zhou, Li Liu, Kate Luby-Phelps, Debabrata Saha, Ralph Mason, **Dawen Zhao**, *UT Southwestern Medical Center, Dallas, TX, USA. Contact e-mail: Dawen.Zhao@UTSouthwestern.edu*

It is well recognized that cancer cells exhibit highly elevated glucose metabolism and up-regulated glucose transporters compared to non-tumor cells. On this basis ^{18}FDG, the glucose analogue, has been used as the most common PET radiotracer to visualize clinical tumors and their metastases. We have recently applied in vivo optical imaging to studying dynamic uptake of a near-infrared dye labeled 2-DG (IRDye800CW 2-DG, Li-Cor Bioscience) by brain tumors in orthotopic mouse xenografts. The orthotopic brain tumor model was established by surgically implanting human glioma U87-luc cells or breast cancer MDA-MB-231-luc cells directly into the right caudal nuclear region of a nude mouse. Intracranial tumor growth was monitored longitudinally by both bioluminescence imaging (BLI) and MRI. When tumor size reached > 5 mm diameter, in vivo fluorescence imaging of IRDye800CW 2-DG was performed. A series of real-time whole body images acquired immediately after i.v. infusion clearly visualized the near-infrared dye circulating into various internal organs sequentially, which validated a capability of the 2-DG dye as a probe to image deep-seated organs or tumors and also provides useful information on the first pass perfusion. Dynamic fluorescent imaging of mouse brain was then performed at different time points. Higher signal intensity in the brain region, but lack of contrast was found between the left (normal) and right (tumor) brain during the first 4 h after injection. However, significantly higher signal intensity was clearly seen in the tumor side of the brain than the contralateral normal side 24 h after injection (tumor/normal ratio 4.1 ± 1.1). In contrast, a control dye, IRDye800carboxyl, showed little difference (ratio 1.4 ± 0.1). These observations may suggest an optimal timing for imaging glucose uptake in clinical brain tumors, which are found to be sometimes indistinct from a high background signal during 18FDG PET, which is necessarily undertaken early due to the short half life of fluorine-18. After in vivo imaging at 24 h, the mice brains were dissected and ex vivo fluorescence imaging performed on whole brain cryosections revealed distinct tumor margins. Moreover, microscopic fluorescence imaging identified cytoplasmic locations of the 2-DG dye in tumor cells. These results further suggest that the near-infrared dye labeled 2-DG may serve as a useful fluorescence imaging probe to assist gross resection of clinical brain tumors. **Acknowledgements**: Supported by DOD IDEA Award W81XWH-08-1-0583 and SAIRP U24 CA126608.

Disclosure of author financial interest or relationships:
H. Zhou, None; **L. Liu**, None; **K. Luby-Phelps**, None; **D. Saha**, None; **R. Mason**, None; **D. Zhao**, None.